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# The Development of Monoclonal Antibodies Against the Cytokinin Zeatin Riboside*

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**Abstract.** Seven monoclonal anti-zeatin riboside antibodies were characterized by radioimmunoassay (RIA) and found to measure femtomole ($10^{-15}$ M) quantities (~20 pg) of this cytokinin. The antibodies had different measuring ranges defined by the linear portion of the logit/log plots; slopes and intercepts of the line varied considerably between the antibodies. Competitive binding trials against *cis*-zeatin riboside (cZR), dihydrozeatin riboside (diHZR), zeatin (Z), and isopentenyl adenosine (iPA) showed differences among the seven antibodies in their cross-reactivities towards these structurally related cytokinins. It was possible to combine selected antibodies to provide a mixture with a predictable measuring range and cross-reactivity; the ability to prepare a highly specific reagent in this manner with well-defined reactivity was noted and differences between monoclonal antibody and polyclonal antiserum probes for measurement of cytokinins were discussed.

Cytokinins play important roles in many plant physiological processes including the regulation of growth and development. Plant hormone research has been hindered by the lack of specific and sensitive methods for qualitative and quantitative assays, in part because cytokinins and other growth regulators

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may be present and active in plants in very small (picomoles) amounts. Ernst et al. (1983) described methods and detection limits for cytokinin analyses as follows: bioassay (20 ng), GC-MS (1 ng), and radioimmunoassay (RIA) (150 pg). Whenham (1983) compared four gas chromatographic detectors for analysis of permethylated cytokinins and found sensitivity limits of 25 pg for a nitrogen-phosphorus detector, 2 ng for flame ionization, 6 ng for an electron capture detector, and 12 ng for a flame photometric detector. Mass spectrometric isotope dilution procedures for cytokinin analysis were described by Summons et al. (1979), but no sensitivity limits were stated.

In recent years, immunoassays that employ polyclonal antibodies have been developed for selected cytokinins (Weiler 1980, Weiler and Spanier 1981, Vold and Leonard 1981). MacDonald et al. (1981) used a combination of high performance liquid chromatography and RIA to measure picogram quantities of certain cytokinins from plant and bacterial sources. In general, the immunological methods are more rapid and sensitive than traditional bioassays or GC-MS techniques. The use of conventional antisera (polyclonal antibodies) for these purposes, however, has the disadvantage that affinities, cross-reactivities, and quantity of antibodies obtained varies with individual animals and even with sera collected at different times from the same animal. Because of these features, no antiserum is precisely reproducible and sera developed in one laboratory are different from those produced in another laboratory.

Cell hybridization techniques are now available to produce large quantities of monoclonal antibodies, mcABs, which can be used as well-defined, highly specific chemical reagents. These antibody-forming hybrid cells—hybridomas—can be cloned and maintained as a culture that produces only one type of antibody. Since the hybridoma cells can be stored in liquid $N_2$ and then recovered, the supply of a particular antibody can be renewed (from the same cloned cell line) and identical antibodies produced in laboratories throughout the world. This paper describes the production and characterization of seven mcABs against the naturally occurring cytokinin, zeatin riboside (ZR). Appropriate characteristics of these antibodies are described to demonstrate the ability for assembly of a mixture of certain mcABs for use as a reproducible serologic reagent in analysis of plant growth substances.

## Methods and Materials

### *Production of Hybridoma Cell Lines*

Groups of BALB/c mice were immunized with ZR conjugated to bovine serum albumin (BSA). Hapten-BSA conjugates were prepared as described by MacDonald et al. (1981) and had a cytokinin:BSA ratio of 15. The immunization schedules consisted of three monthly i.p. injections of 300 μg of conjugate in 0.15 M phosphate-buffered saline, pH 7.2, (PBS) emulsified in Freund's Complete Adjuvant per injection. Sera collected from immunized animals were assayed for anti-ZR antibodies with an enzyme-linked immunosorbent assay (ELISA) and RIA to insure that adequate immune responses to the hapten had occurred. Four days prior to splenocyte removal the mice were boosted (i.p.)

with 300 μg of protein-hapten conjugate in PBS and on the following day, each animal received an intravenous injection of 100 μg of the conjugate in PBS.

The cell fusion method of Oi and Herzenberg (1980) was used to derive hybridomas that secreted anti-ZR mcAbs. Splenocytes harvested from hyperimmune BALB/c mice were fused with Sp2/o mouse myeloma cells in 50% polyethylene glycol 1,000–10% dimethyl sulphoxide, and plated into 96-well culture plates. After 24 h, HAT medium (Littlefield, 1964) was added to each well to select for growth of hybrid cells. The cells were maintained in HAT medium until macroscopic hybrid growth was apparent, at which time the HAT was replaced by HT medium (Oi and Herzenberg, 1980), and eventually by Dulbecco's modification of Eagle's medium (Flow Labs, Inc., McLean, Virginia) plus 10% fetal bovine serum (DMEM/10).

*Enzyme-Linked Immunosorbent Assay*

An ELISA was developed to detect anti-ZR antibodies in mouse antisera and in hybridoma culture fluids and was conducted in the following manner. Wells of a 96-well culture plate were coated with 100 μl/well of ZR conjugated to ovalbumin (75 μg/ml) (MacDonald et al. 1981), incubated overnight at 5C, emptied, washed three times with PBS-Tween (PBS contained 0.05% Tween 20), and then "blocked" with 100 μl of 1% ovalbumin in PBS (37C, 30 min) to prevent non-specific protein adsorption. After the plate was washed twice with PBS-Tween, 100 μl of hybridoma culture fluid or mouse antiserum were added to the appropriate wells, the plate was incubated at 37C for 60 min, and then washed three times with PBS-Tween. Each well then received 100 μl of a 1:1000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (H&L chains) (Sigma Chemical Co., St. Louis, MO) and the plate was incubated for an additional 60 min at 37C. After three washes with PBS-Tween, 100 μl of substrate solution (3 mg/ml of p-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, 0.5 mM $MgCl_2$) were added to each well and the plate incubated at 37C for 60 min. The color reactions in each well were determined by measurement of the absorbance at 405 nm by an enzyme immunoassay plate reader.

*Radioimmunoassay*

These assays were conducted according to methods described by MacDonald et al. (1981). This same procedure was used for the cross-reactivity studies which used certain cytokinins over the range of 0.05–15 pmoles as unlabeled competitors. After specified amounts of each inhibitor were used in an RIA, B/Bo vs. picomoles was plotted to determine the number of picomoles required to give a B/Bo ratio = 0.5. Percent cross-reactivities were then expressed as

$$\frac{\#\text{pmol unlabeled ZR for B/Bo} = 0.5}{\#\text{ pmol unlabeled inhibitor for B/Bo} = 0.5}$$

where Bo = the number of counts of labeled ZR bound by the antibody when no competitor was present, and B = the number of counts of labeled ZR bound in the presence of the selected competitor.

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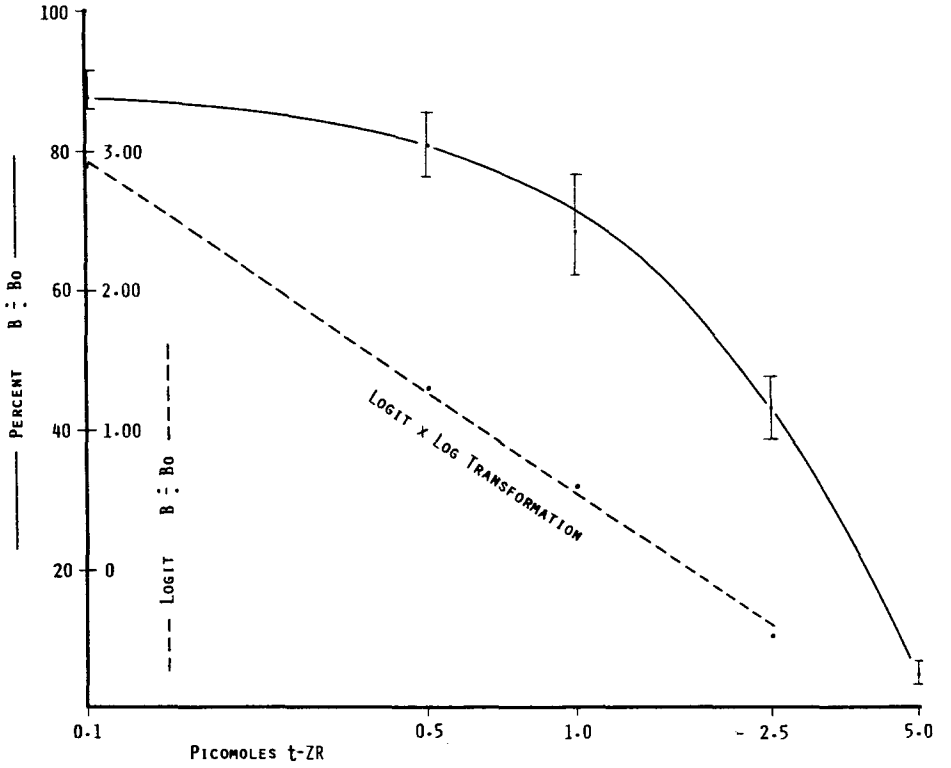


**Fig. 1.** Representative competitive-binding radioimmunoassay of monoclonal antibody 16 in which unlabeled zeatin riboside (ZR) acted as a competitor with $^{3}$H-ZR dialcohol.

cross-reactivities of these two antibodies with other cytokinins (mcAB 5 had greater than 70% cross-reactivity with *cis*-zeatin riboside [cZR], dihydrozeatin riboside [diHZR], and zeatin [Z] while mcAB 7 had from 25–39% cross-reactivity with these same ligands).

Subisotype determinations indicated that all seven of the characterized mcABs were IgG molecules. Two of them, mcABs 3 and 5 were IgG2a while the remainder (mcABs 7, 8, 10, 12, and 16) were identified as IgG1.

## Discussion

The anti-ZR mcABS developed in this study provided very sensitive probes for this cytokinin in RIAs; in general, sensitivity limits extended as low as 50 femtomoles (~20 pg). This sensitivity is similar to that noted in other studies which used rabbit anti-ZR sera in RIA (Badenoch-Jones et al. 1984, Weiler 1980, Weiler and Spanier 1981, Vold and Leonard 1981) and in an enzyme immunoassay (Hansen et al. 1984). Each mcAB had a rather narrow measuring range for ZR defined by the slope of the line of the logit/log plot of B/Bo against picomoles of unlabeled ligand present in the RIA; lines with steeper slopes presented a narrower range of linear measurement. The restricted measurement ranges found in this study are in contrast to rather wide linear ranges

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eatin riboside (ZR) served as
of seven monoclonal anti-ZR

| l)<br>error | Linear range<br>(pmol ZR) |
|---|---|
| 5 | 0.05–2.50 |
| 1 | 0.05–2.50 |
| 4 | 1.00–5.00 |
| 8 | 0.05–2.50 |
| 5 | 0.05–2.50 |
| 9 | 1.00–5.00 |
| 2 | 0.10–5.00 |

ice of an unlabeled competitor
r (B) were transformed as logit
f pmol of unlabeled competitor

competitive-binding of selected
-zeatin riboside antibodies.[a]

| Zeatin | iPA[c] |
|---|---|
| —[f] | — |
| 0.5 – 10.0 | — |
| 1.0 – 10.0 | — |
| 1.0 – 10.0 | 1.0 – 12.5 |
| — | 0.5 – 5.0 |
| — | 1.0 – 5.0 |
| 1.0 – 10.0 | — |

hybridoma culture fluid to bind
nce of selected amounts of the
the presence of the inhibitor (B)
was plotted as ln (B/Bo)/(100 –

ed (up to 15 pmol).

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an essential difference be-
hile a mcAB represents a
ing affinity and specificity,
olecular species, each with
ly, this population of anti-
cific ligand since each mo-

**Table 3.** Percent cross-reactivities of seven monoclonal anti-zeatin riboside antibodies with certain structurally related cytokinins and purine compounds.[a]

| Monoclonal antibody | ZR[b] | cZR[c] | diHZR[d] | Zeatin | iPA[e] | Adenosine | N-6-B[f] |
|---|---|---|---|---|---|---|---|
| #3 | 100 | 41 | 67 | —[g] | — | — | — |
| #5 | 100 | 48 | 52 | 57 | — | — | — |
| #7 | 100 | 39 | 65 | 28 | — | — | — |
| #8 | 100 | — | 52 | 30 | 22 | — | — |
| #10 | 100 | — | 56 | — | 28 | — | — |
| #12 | 100 | 72 | — | — | 221 | — | — |
| #16 | 100 | — | 15 | 22 | — | — | — |

[a] Selected compounds were used in competitive-binding radioimmunoassays to determine the number of picomoles of each required to provide a B/Bo value = 0.50. Percent cross-reactivity was determined as follows: (#pmol of unlabeled ZR required for B/Bo = 0.50)/(#pmol of competitor required for B/Bo = 0.50) × (100).
[b] Zeatin riboside.
[c] *cis*-zeatin riboside.
[d] Dihydro-zeatin riboside.
[e] Isopentenyl adenosine.
[f] N-6-benzyl adenine.
[g] "—" indicates that cross-reactivity could not be determined because no linear range of antibody-competitor reaction occurred in the ranges of the competitor tested (up to 15 pmol).

**Table 4.** Linear ranges (pmol) for measurement of selected cytokinins by a mixture of two anti-zeatin riboside monoclonal antibodies.

| Monoclonal antibody | Linear measurement range (pmol) for specified cytokinin | | | |
|---|---|---|---|---|
| | cZR[b] | r[c] | Zeatin | r |
| #3 | 0.50 – 10.0 | −0.99 | —[d] | — |
| #16 | — | — | 1.0 – 10.0 | −0.97 |
| #3 & #16[a] | 0.50 – 10.0 | −0.98 | 1.0 – 10.0 | −0.98 |

[a] A mixture which consisted of equal amounts of each antibody (as measured by the ability to bind 50% of the cpm of $^3$H-ZR in radioimmunoassay) was used in competitive binding experiments in which unlabeled cis-zeatin riboside or unlabeled zeatin competed with $^3$H-ZR for antibody binding sites.
[b] *cis*-zeatin riboside.
[c] Linear correlation coefficient (significant at $P \leq 0.001$).
[d] No linear measurement range.

lecular species contributes a unique affinity and measuring range to that of the whole antiserum.

This study developed seven different mcABs against the cytokinin ZR and characterized their reactivities with certain similar molecules. In addition, two of these antibodies were combined to provide a reagent with a very specific measurement range and cross-reactivity; it should be pointed out that this mixture of mcABs was not assembled to mimic a polyclonal anti-serum, but rather to act as a predictable and reproducible serologic reagent. Our obser-

vation that a mixture of two antibodies could be prepared with predictable cross-reactivity was both qualitatively and quantitatively as expected from previous characterization of the individual antibodies.

The cross-reactivity of these anti-ZR antibodies with other cytokinins was similar to that noted by Badenoch-Jones et al. (1984), Weiler (1980) and Weiler and Spanier (1981) with polyclonal anti-ZR and anti-isopentenyl adenosine (iPA) sera, although certain mcABs in this study showed higher cross-reactivity with cZR and diHZR than noted by previous authors (Table 3). Woodsworth et al. (1983) prepared mcABs against iPA; certain of these antibodies were highly specific for iPA while others cross-reacted with selected structurally related molecules. The enhanced cross-reactivity of mcABs as compared to polyclonal antiserum is well documented for other antigens (Lane and Hoeffler 1980, Pillemar and Weissman 1981, Dulbecco et al. 1981) and possible explanations have been offered (Lane and Koprowski 1982). The ability to prepare mixtures of mcABs may be very useful for specific removal of selected cytokinins from a plant extract for subsequent analysis on HPLC or similar instrumentation as suggested by MacDonald et al. (1981). Alternatively, preparation of mcABs with high specificity, such as those described by Woodsworth et al. (1983) may allow direct quantitation of cytokinins in plant extracts without the use of additional analytical techniques.

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